LABORATORY GUIDE – DIAGNOSTICS FOR PHYTOPHTHORA RAMORUM

A. INTRODUCTION

This guide to laboratories is designed to outline basic components (facility, equipment, and standard procedures) needed to process and report sample results using the current USDA approved protocols for *Phytophthora ramorum* diagnostics. These guidelines are intended to complement the protocols listed on the USDA-APHIS-PPQ website, http://www.aphis.usda.gov/ppq/ispm/pramorum/. The function of this guide is to facilitate the ability of non-USDA laboratories to provide officially sanctioned diagnoses for programs under the amended Federal Order for *Phytophthora ramorum* (list the link to the FO). The components outlined in this guide are deemed to be the minimum requirements needed to sustain a successful *P. ramorum* diagnostic program for the USDA emergency program.

B. GENERAL CONSIDERATIONS

All of the components and procedures described in this guide attempt to facilitate the twofold goal of the *P. ramorum* diagnostic procedures; ability to achieve reliable test results and prevention of sample contamination. Each goal must be completely accomplished to provide reliable and usable results for the USDA emergency program. Of the two goals, prevention of contamination must be continuously applied throughout the diagnosis.

B.1. Contamination of Samples

Contamination of samples would prevent reliable retesting of samples. Cross-contamination of samples can occur in either ELISA, culture ID, or nested PCR from the initial point of contact when the sampling takes place, at any point where the sample is moved or handled, during purification of the nucleic acid, during each stage of the nested PCR, and finally lane to lane during the gel analysis of the PCR test result. Because the components being detected are different for each test, steps that prevent cross-contamination for one assay (such as culturing or ELISA) may not be sufficient with others (like nested PCR).

The cautionary steps needed to prevent sample contamination in labs performing PCR diagnostics makes successful diagnosis of *P. ramorum* more complicated than what is typically required in most plant diagnostic labs. Each diagnostic stage must be physically separated from the previous stage to reduce the risk of contamination. Although encouraged but not required by the USDA, any lab that wishes to attempt culturing the organism will need to provide a separate room to ensure pathogen isolation from incoming samples and other diagnostics tests. These extra components will be covered in a separate section.

Although the vast majority of samples assayed in the lab for *P. ramorum* will not have to undergo molecular testing, many will be tested using the nested PCR and the precautions described throughout this guide will help generate reliable test data used for regulatory purposes. General precautions that should be taken are included in sections C.3., C.4., C.5., and C.6.

B.2. **Sampling Procedures**

The sampling procedure for *P. ramorum* is outlined in the USDA-APHIS-PPQ website within the 'Trace Forward Protocol' (list the web link) and includes how the information that should be recorded for each sample, how the sample should be taken, how the sample is to be stored prior to shipment, and how the sample is to be shipped. Additional information on decontamination between sampling sites is included. All diagnostic lab personnel should be familiar with the sampling procedure so that they are aware of treatment of the sample prior to its arrival in the lab. The sampling is usually not performed by diagnostic lab personnel, but by trained field inspectors. Personnel involved in field collections should never enter the diagnostic testing laboratory because of potential *P. ramorum* contaminants on clothes, shoes, hair, and other personal items. Lab personnel that must be involved in field sampling will need to take extra precautions to prevent introducing contaminants into the lab.

The first contact that most lab personnel will have with the samples is when shipments are delivered to the lab. If sample parcels are received by office personnel, they should be made aware of potential arrivals so that parcels are treated correctly upon reception. At the point of shipment reception, a log should note the time of arrival, who the package was from, who received the package, and the shipper with tracking number. The parcels should remain unopened and secured in a cool, dry place away from direct sunlight, and a phone call made to a trained member of the diagnostic testing lab to receive the package to continue chain of custody. The trained lab personnel (receptionist or technician) should take the parcel directly to the lab for processing. The package should be opened according to the procedures outlined in the state and federal pest permit for *P. ramorum*, and will include as a minimum that the samples should remain isolated from the natural environment outside the lab facility until a final disposition is reached. At the end of testing and once a final disposition is reached, the samples and packaging should be disposed of appropriately according to permit conditions and will likely include autoclave destruction of the plant samples and all packaging materials.

B.3. Basic Infrastructure Requirements

The rooms needed for diagnosis are: a receiving room that should contain a Class II, Type A bio-safety cabinet, a storage room or dedicated refrigerator with the capability of being locked or not available to the public, a sample processing room, a DNA preparation/sample extraction room, (ideally) a separate PCR preparation room or PCR WorkStation™, a small area for the PCR machines physically separated (not in the same room) from the PCR set-up area, and a separate room for PCR gel analysis. PCR machines and PCR products should never enter the area where samples are taken, prepared, or where PCR reaction components are diluted or distributed. *P. ramorum* culturing at the same lab would require at least one more room with a transfer hood for isolation and growth of the organism. All of the rooms should be well ventilated and physically separated enough to prevent airflow from one area to flow into others. Ideally, personnel who sample should be different than those who process the DNA. If this is not possible, steps should be taken between the two processes to minimize possible DNA contamination, such as changing lab coats and putting on other 'clean room' apparel. Personnel should not be changing from one area to others several times a day (see additional comments in B. above).

C. GENERAL CONSIDERATIONS FOR THE TESTING LAB

The following general guidelines for the testing lab will cover the six basic areas of diagnostic activity for each sample. The areas deal with sample reception and log-in, storage of samples, sample selection and initial processing, ELISA, DNA extractions, and PCR set-up and analysis.

C.1. Organization and Log-in of Samples

Following delivery, the diagnostic laboratory should be immediately notified and trained lab personnel sent to claim the package and take it to the sample receiving/log-in room of the lab. The package should be opened according to permit instructions in an approved and certified bio-safety cabinet. The reasoning behind opening parcels in a separate room is to minimize the chance of contaminating uninfected samples from other shipments if an infected sample container is leaking or broken. The package contents should be examined to determine if the appropriate paperwork accompanies each sample. Sample numbering and paper work should match exactly. Samples that are degraded or in poor condition, or packages with broken or leaky sample bags should be rejected, and disposed of by autoclaving immediately. In practice, episodes of package contamination should be rare, especially if the samples are double-bagged as called for in the sampling protocol described above. Degraded samples, experience has shown, produce poor nucleic acid and may yield false negative results. Sample rejection and discrepancies between the sample and accompanying paperwork should be resolved by calling the sender immediately to resend a duplicate sample or clarify paperwork. Information to be recorded at sample log-in includes the delivery source, date parcel sent, condition of outer package, and condition of inner packages (noting the presence of leaks or breakage), condition of the tissue, and any request of the sender for a duplicate sample. The sample receiving room should be clean with adequate bench space for organizing samples and paperwork. A traditional plant diagnostic lab sample preparation room *could* be used if no *P. ramorum* cultures have been isolated and grown there.

C.2. Storage of Samples

After samples are determined to contain the appropriate matching paperwork and are in good condition, the appropriate trained lab personnel are now responsible for the chain of custody of the sample that began at sample reception and will conclude with reporting test results and destruction of the sample and packaging materials. The samples can be processed immediately or repackaged for storage in a refrigerator or a cold room at about 4ºC that is not used to store cultures of *P. ramorum*. Storage conditions should be conducive to preserving the integrity of the samples; however tissue symptomatic for *P. ramorum* infection may degrade faster than non-symptomatic tissue rendering the sample unusable and if used may produce false results.

C.3. Initial processing

The initial processing should be conducted in a room separate from both the initial examination and the following extraction and PCR analysis procedures. A Type II, Class A Bio-safety cabinet should be used for this purpose. The hood needs to be properly prepared before and after sampling by wiping out any residue with a 10% household bleach solution or 70% ETOH followed by running the UV sterilization lamps for fifteen minutes. Medical quality matting paper should be laid down in the hood and samples are cut in weigh boats changed between each sample. Gloves must be worn to select leaves and cut samples and changed between each sample to avoid contaminating the next sample. Mycelium and spores from *P. ramorum*-infected leaves can be transferred from infected leaf sets to healthy leaf sets and may result in a false positive PCR test. A good rule of thumb is to treat each sample as if it is infected and as if the next sample to be processed is healthy and take measures to minimize contamination of that next sample. Cutting implements should either be disposed of between samples, or for cork borers, scalpels, or other non-disposable cutting implements, dipped in ethanol and flamed between samples to avoid direct contamination.

Leaf tissue for DNA extraction should be collected from the margin area between the necrotic lesion and healthy tissue of symptomatic leaves. Enough tissue should be taken as a sub-sample to serve both ELISA and the nested PCR assay if needed. Use of 15 ml falcon tubes for the processed sample might be good at this point. The outside of the tube should be wiped down with a sterile cloth to minimize plant material that may have stuck to the outside of the tube prior to transfer to a rack or bag. Alternatively, sub-samples could simultaneously be selected out for immediate ELISA processing at this time. However, it is still important to generate a 'back-up' sub-sample, especially if you wish to attempt culturing the main sample later.

C.4. ELISA

ELISA sub-samples should be selected and prepared as directed in the ELISA protocol on the USDA-APHIS-PPQ website. The document on this site outlines the procedure and the basic equipment needs as well as directs the reader to the Agdia Inc. website for additional background information on the test. Generally, an area set aside for sample grinding with running water and a lab bench in a room with a sink is sufficient for ELISA. This ELISA is a sensitive assay, so care must be taken to insure that areas used for ELISA are not contaminated with *P. ramorum*. These rooms should also not be used for routine *Phytophthora* or *Pythium* testing (for example: routine potato late blight or seedling damping off) as this ELISA reacts with many of these organisms. Overwashing and underwashing ELISA plates can affect the outcome of the test. Make sure that any uncertainty in performance of the ELISA is discussed with Agdia. Any questions regarding the procedure provided in the kits from Agdia should be directed at Agdia, Inc. (1-800-622-4342). Any problems in the performance of the kit and controls should be reported to Agdia immediately.

C.5. <u>DNA extraction</u>

Only ELISA positive samples need to be further tested by PCR. ELISA negative samples are considered negative for *P. ramorum* by the USDA. Further testing of ELISA positives requires sorting and sampling of the previously generated sub-samples for DNA extraction. The recommended method for DNA extraction using the USDA-APHIS-PPQ protocol is the Qiagen DNeasy Plant Mini kit, although another more labor intensive protocol is also currently available. For DNA extraction using the DNeasy Plant Mini kit, the maximum amount of tissue used is 100 mg fresh wt. For many of the plant species that are hosts to P. ramorum including Rhododendron, Camellia, Bay Laurel, *Syringa*, and *Viburnum*, 9-10 leaf discs using a No. 3 cork borer or 15-16 of the 5mm x 5mm cut leaf pieces is about 100mg. Leaves from other host species might need to be weighed out to determine how much is needed for DNA extraction. This sub-sampling takes about five minutes per sample, depending on the quality of the samples and the ability to find suitable suspect areas for sub-sampling. It is recommended to sample close to sites of previous sampling for ELISA thus ensuring more consistency of the results. There is important information and techniques that can be employed to reduce contamination on the USDA-APHIS-PPQ website describing the PCR protocol.

Samples in 100 mg aliquots placed in 1.5 ml microcentrifuge tubes are now ready for DNA extraction. This procedure should take place in a ‘clean lab’, a standard molecular biology equipped lab not exposed to plant material or microbial cultures. Equipment needed for DNA extractions should include: a full set of standard micropipettes with appropriate aerosol-resistant barrier tips, autoclaved microcentrifuge tubes, microcentrifuge (see below), liquid nitrogen, mini-bead beater or appropriate tissue maceration system, sterile distilled water, reagent grade ethanol, ice bucket, refrigerator and freezer. The DNA extraction protocol is on the USDA-APHIS-PPQ website and part of it is presented here to facilitate setting up a lab for DNA extraction.

“In order to avoid cross contamination please designate separate rooms or lab areas for each segment of the work and use separate designated sets of pipettes. Use aerosol-resistant barrier pipette tips. Centrifuge any DNA-containing tubes before opening so that any liquid near the rim of the tube is removed; centrifuge rotors designed for aerosol containment are recommended. Always use microfuge tube openers to avoid direct contact with the top rim of the microfuge tubes. It is a good practice to store plant samples or extracts in a separate freezer or freezer compartment from PCR reaction components. If leaf samples are contaminated with soil rinse them in sterile water and pat-dry them with a hand towel before sampling. Wear gloves and change them often, particularly between different segments of the DNA extraction procedures. Use disposable lab mats to cover bench areas and change them between each set of extractions. Never autoclave any *P. ramorum*–contaminated plant material, culture plates, or soil in an autoclave used to sterilize buffers, glassware, or plasticware used in the SOD DNA extraction because of potential contamination from aerosols within the autoclave. All racks, tube openers, and other plastic materials used in the procedure should be decontaminated between each set of extractions by soaking in a 10% bleach solution (a 1:9 dilution of commercial bleach in water) for a minimum of 30 minutes followed by two rinses with water to remove the bleach solution. Bench areas, pipettes, centrifuge rotors, lab chairs, drawer handles, and other knobs, etc. in the environment of the bench used to do DNA extractions should be wiped down every couple of days with a DNA elimination solution such as DNA Away."

C.6. PCR set-up

Ideally, the PCR should be done in a separate room than the DNA extraction, especially if aerosol-controlled microcentrifuges are not used. Minimum requirements are that the PCR should be set up on a lab bench that has been cleaned and the bench paper replaced if it is also used for DNA extraction. Another option is using clean bench-top PCR enclosure cabinets. In addition, it is also essential that a dedicated set of pipettes (*PCR-only*) that are not used for DNA extraction be used to set up the PCR. All preparation of stock solutions should occur as far away from any samples (including positive controls) as possible, preferably in a laminar flow hood. Storage of all PCR reagents should be in refrigerators and freezers not used for samples. Adding DNA templates to the PCR reactions should be done outside the clean PCR area using a dedicated pipette not pipettes used for making and aliquoting the PCR mix or involved with post PCR analysis.

The basic equipment for PCR set up includes a standard molecular biology lab with a dedicated reagent refrigerator and freezer, a thermocycler (the USDA protocol uses a T-gradient from Biometra, but others could be used pending a few tests to confirm compatibility with the protocol), thin-wall PCR disposable tubes for the thermocycler, dedicated sets of pipettes, sterilized pipette tips, commercially synthesized primers (such as IDT Technologies) and Platinum *Taq* Polymerase from Invitrogen (cat. No 10966-034). A source of crushed ice and appropriate racks for the PCR tubes are also necessary.

Analysis of the amplified (PCR) products is done by electrophoresis. The standard equipment includes: a gel running box with appropriate gel forms and combs, microwave oven, DC electrophoresis power supply, a UV lightbox, a camera or gel documentation system outfitted with the appropriate filters and with the resolution requirements of reading a gel and recording the results, and necessary glass- or plastic-ware. Gel running buffer (usually TAE), agarose, ethidium bromide or another DNA stain which are all commercially available. Electrophoresis should be run in a separate room from any of the PCR processes and using a separate set of pipettes with aerosol-resistant barrier pipette tips to avoid carry-over contamination of PCR products. **Never bring PCR products to any area where PCR reactions are prepared**.

Successful completion of the nested PCR in the PCR protocol, where the appropriate negative and positive controls react as expected, require two successive reactions. An additional PCR reaction needs to be performed to make a final negative determination. This assay (the multiplex PCR) uses universal primers as an additional control to insure that the isolated DNA is of sufficient quality and quantity for assays.

D. GENERAL LIST OF EQUIPMENT/PERSONNEL NEEDED

Initial Receipt at Lab
- -appropriate storage
- -office personnel (aware of deliveries and informs staff)

Organization and Log-in
- -Trained member of the diagnostic lab
- -dry lab
- -disinfection supplies

Storage
- -cold room or dedicated refrigerator

Sub-sampling
- -isolated room with biosafety cabinet
- -disinfection and implement flame source
- -disp. gloves, Kim wipes
- -sterile forceps, single-edge razors or scalpel
- -laboratory personnel trained in sterile techniques with additional training in avoiding DNA contamination

ELISA
- -standard wet lab, separate grinding and assay areas
- -plant tissue maceration system, (appropriate cleaning of system between samples if necessary).
- -ELISA plates, plate washing supplies, ELISA plate reader
- -Agdia kit
- -laboratory personnel trained in ELISA

DNA Extraction

-isolated clean lab set up for molecular biology

-standard set of calibrated micropipettes, microcentrifuge, source of liquid nitrogen, plant tissue maceration system, disposal aerosol barrier tips, sterile microcentrifuge tubes, reagent-grade ethanol, Qiagen DNeasy Plant Mini Kit, disp. gloves, lab coat, decontamination supplies.

-laboratory personnel proficient in molecular biology (with additional specific training for SOD).

Polymerase Chain Reaction

-molecular biology lab away from DNA extraction area (this could be a separate lab bench, but ideally a separate room)

-standard set of calibrated micropipettes (different than ones used for DNA extraction), disposal aerosol barrier tips, sterile microcentrifuge tubes, thin-wall PCR tubes, disp. gloves, lab coat, decontamination supplies.

-appropriate thermocycler

-laboratory personnel proficient in molecular biology (with additional specific training for SOD).

Gel Electrophoresis and Documentation

-can be part of molecular biology lab or photographic dark room

-standard or commercial gel electrophoresis equipment and power supply

-gel staining equipment and appropriate safety and disposal supplies

-gel documentation or photography equipment

-laboratory personnel proficient in DNA extraction and/or PCR, and trained in gel electrophoresis

-trained and authorized laboratory personnel to read results and record determinations